Sodium 4-phenylbutyrate inhibits protein glycation

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Abstract. The production and accumulation of advanced glycation end-products (AGEs) are hypothesized to have a causal role in the development of the complications associated with aging and lifestyle-related diseases, such as diabetes, atherosclerosis and hyperlipidemia. Therefore, it is important to reduce the production and accumulation of AGEs. In the present study, the ability of sodium 4-phenylbutyrate (PBA) on inhibition of glycation was assessed. *In vitro*, PBA inhibited the glycation of albumin and collagen by up to 42.1 and 36.9%, respectively. Furthermore, when spontaneously diabetic KK mice were administered PBA (20 mg/day) or vehicle orally, glycosuria developed rapidly in the control mice, but after 6 weeks, only one treated mouse was glycosuric. In addition, the weight gain and HbA1c levels were significantly lower in the treated mice compared with the untreated mice (weight gain, 36.0 g vs. 39.4 g, P<0.01; HbA1C level, 3.96 vs. 4.78%, P<0.01; respectively). These results suggested that PBA also inhibited glycation *in vivo*. Further studies are required to determine whether PBA may be effective for the therapy or prevention of aging or lifestyle-related diseases caused by the accumulation of AGEs. The method of administration and the side-effects of PBA have already been established as PBA is already used clinically. Therefore, the repurposing of PBA for reducing AGE levels may be a potential option to reduce complications associated with aging.

Introduction

Glycation is a non-enzymatic chemical reaction that occurs between a ketone or aldehyde group of fructose or glucose and an amino acid residue or the hydroxy-group of a protein or lipid, and is often referred to as the Maillard reaction. Protein glycation occurs through a complex series of very slow reactions in the body, including the formation of the stable Amadori-lysine products (Schiff bases). These give rise to advanced glycation end-products (AGEs) (1-4).

It is hypothesized that the production and accumulation of AGEs have causal roles in the development of the complications associated with aging and lifestyle-related diseases, such as diabetes, atherosclerosis and hyperlipidemia (1-4). Furthermore, the production and accumulation of AGEs are involved in the development of other diseases, such as cardiovascular diseases, cerebrovascular disorders, chronic renal failure, Alzheimer's disease and Parkinson's disease (5-9). Therefore, the identification of safe treatments that can inhibit glycation is required, as they may exhibit anti-aging effects, or serve as a therapeutic option for prevention of diseases associated with glycation (1,10).

In the present study, sodium 4-phenylbutyrate (PBA) was assessed as a potential candidate for use as an anti-glycation agent. PBA is an aromatic fatty acid that acts as a histone deacetylase inhibitor, ammonia scavenger and chemical chaperone (11,12). It is currently used as a treatment of urea cycle disorders, as it can promote the excretion of residual nitrogen (13), and is the subject of clinical trials for use as a treatment of several other diseases (14,15). Recently, PBA has been shown to possess potent anti-oxidative effects that are achieved via the suppression of endoplasmic reticulum stress, as well as an anti-inflammatory effect, which is exerted through nuclear factor-κB (NF-κB) (16-18).

It was previously reported that PBA may be effective for the treatment of neurodegenerative diseases, including Parkinson's disease, and it can suppress the onset of dextran sulfate sodium-induced colitis (19-21). Furthermore, previous studies have suggested that PBA is effective against diabetes mellitus and hyperlipidemia (16,22). Importantly, treatment with PBA is associated with very few side effects (13,15,19-21).

There are no reports assessing the anti-glycation effects of existing drugs, to the best of our knowledge. Therefore, the aim of the present study was to determine whether PBA inhibited the glycation of proteins *in vitro* and *in vivo*.

Materials and methods

Effect of PBA on the glycation of albumin. The glycation of albumin was measured *in vitro* at the Body Support Institute

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Abbreviations: AGE, advanced glycation end-product; PBA, sodium 4-phenylbutyrate; HbA1c, hemoglobin A1c

Key words: AGE, PBA, glycation inhibition, albumin, collagen, KK mouse, lifestyle-related disease

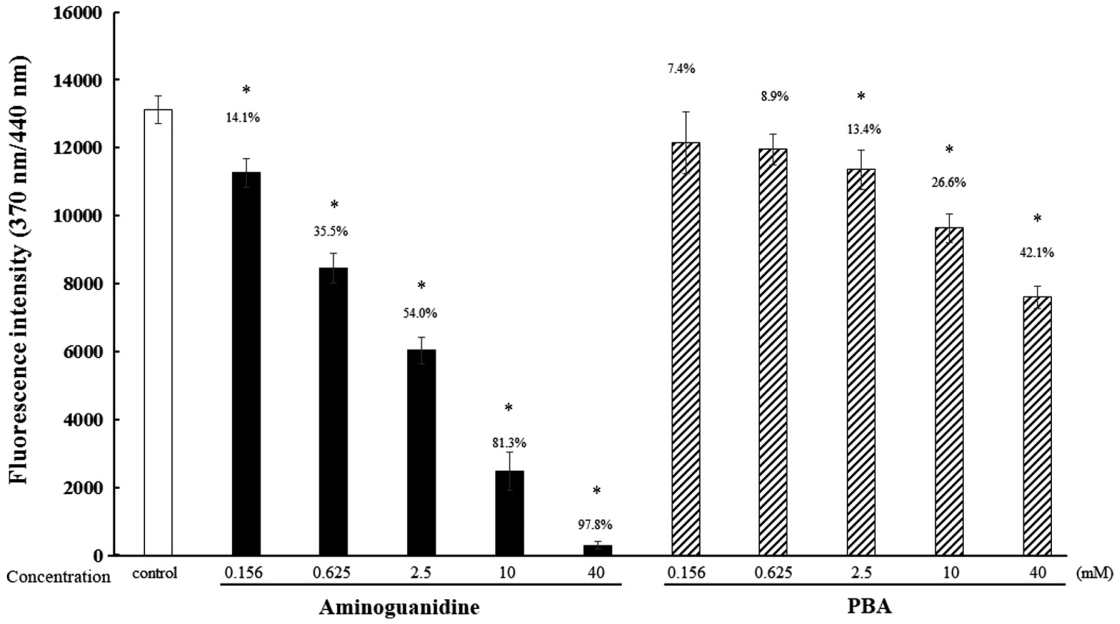

Figure 1. Inhibitory effect of PBA on albumin glycation. The vertical axis shows the fluorescence intensity. The number above each bar is the percentage reduction vs. Control. Data are presented as the mean ± standard error of the mean, and were analyzed using a one-way ANOVA, followed by Dunnett's post-hoc test. n=6. *P<0.01 vs. Control. Control, Dulbecco's PBS only; PBA, sodium 4-phenylbutyrate.

(ARKRAY Karada Lab; ARKRAY, Inc.) (23). Briefly, the α-glucose concentration was adjusted to 0.2 mol/l and the human serum albumin (cat. no. A-1887, Sigma-Aldrich; Merck KGaA) concentration was adjusted to 8 mg/ml using Dulbecco's PBS (DPBS; Nacalai Tesqe, Inc.). Subsequently, PBA (LKT Laboratories, Inc.) and the positive control, aminoguanidine (FUJIFILM Wako Pure Chemical Corporation) were added at a range of concentrations. After incubation at 60˚C for 40 h, the concentrations of the AGEs produced were quantified by measuring the fluorescence intensities of the solutions (excitation wavelength, 370 nm; emission wavelength: 440 nm) using a microplate reader (Infinite F200 PRO, Tecan Group, Ltd.). The experiment was performed three times, in duplicate (n=6).

Effect of PBA on the glycation of collagen. The glycation of collagen was measured using a Collagen Glycation assay kit: Glyceraldehyde (cat. no. AK71; Cosmo Bio., Co., Ltd.), according to the manufacturer's protocol. Briefly, the neutralized collagen solution was cooled and 50 μl was carefully added to each well of a 96-well plate, while maintaining the temperature at <10˚C. Next, the plate was incubated overnight at 37˚C in a humidified atmosphere. Then, PBA, aminoguanidine in DPBS and DPBS alone (as the negative control) were sterilized by filtering using 0.22-μm filters, and 40 μl of each solution was added to the collagen gel. Finally, 10 μl 500 mM glyceraldehyde was added to each well and the contents of the wells were mixed using a plate mixer (Iwaki; AGC Techno Glass Co., Ltd.). After incubation for 24 h at 37˚C in a humidified atmosphere, the concentrations of AGEs was assessed by measuring fluorescence intensity (excitation wavelength, 370 nm; emission wavelength, 440 nm) using a microplate reader. The experiment was repeated three times in duplicate (n=6).

Effect of PBA on glycation in KK mice. For the *in vivo* experiments, 10-week-old male KK/Ta Jcl mice (KK mice) weighing ~30 g were purchased from CLEA Japan (CLEA Japan, Inc.). Mice were housed individually in cages in an animal holding room with a 12 h dark/light cycle at 20±5˚C. The mice were divided randomly into two groups: Untreated control group (n=5) and a 20 mg/day PBA-treated group (n=5). PBA was administered orally at a concentration of 20 mg/200 μl H_2O once daily, and 200 μl H_2O was administered to the control mice. The doses used were based on a previous study (21), and equivalent to the doses administered to humans in existing drug preparations, such as Buphenyl (14,15,19-21). The mice were treated for 8 weeks from 10 weeks of age. Their body mass and urine glucose levels were measured every 7 days, and their HbA1c levels were measured every 14 days by obtaining blood from the tail vein (~1 μl) using a HbA1c measuring device (DCA Vantage; Siemens Healthineers). Glycosuria was identified in the urine using a dipstick (cat. no. UA-P1G5; Terumo Corporation). Blood glucose was measured in ~1 μl blood obtained from the tail vein using a blood glucose meter (Glutest ai; Sanwa Kagaku Kenkyusho Co., Ltd.). The urinary albumin concentration was analyzed using an Lbis® Albumin Mouse ELISA kit (FUJIFILM Wako Pure Chemical Corporation), according to the manufacturer's protocol. The mice were fed standard laboratory chow and provided with water *ad libitum*. Their food intake was measured every 7 days by measuring the mass of food remaining in each cage after 24 h. At the end of the experiment, the mice were euthanized by cervical dislocation after anesthesia by isoflurane inhalation. The animal experiments were performed in accordance with Fukuoka University guidelines and were approved by the Ethics Committee for Animal Care and Use of Fukuoka University (approval no. 1909069).

Statistical analysis. Statistical analysis was performed using GraphPad Prism version 6 (GraphPad Software, Inc.). Data are presented as the mean ± standard error of mean. Data were

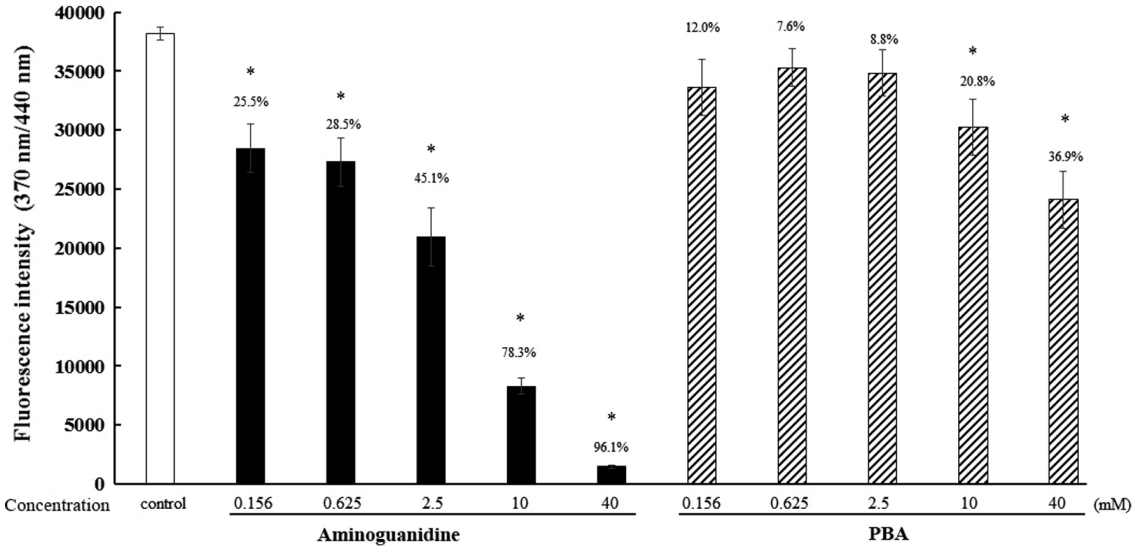

Figure 2. Inhibitory effect of PBA on collagen glycation. The vertical axis shows the fluorescence intensity. The number above each bar is the percentage reduction vs. Control. Data are presented as the mean ± standard error of the mean, and were analyzed using a one-way ANOVA, followed by Dunnett's post-hoc test. n=6. *P<0.01 vs. Control. Control, Dulbecco's PBS only; PBA, sodium 4-phenylbutyrate.

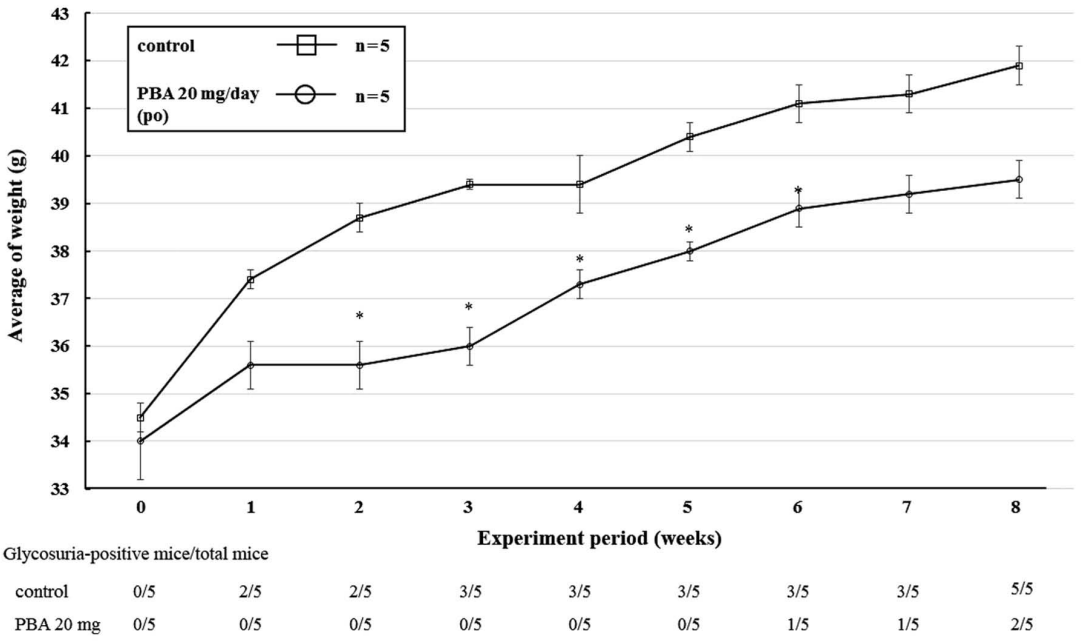

Figure 3. Effect of PBA on the body mass of KK mice. The vertical axis shows the body mass of each group and the horizontal axis shows the number of weeks of the study elapsed. The number of mice which were glycosuria positive at each time point is shown underneath the graph. Data are presented as the mean ± standard error of the mean, and were analyzed using a two-way ANOVA. n=5. *P<0.01 vs. Control. Control, H_2O; PBA, sodium 4-phenylbutyrate; po, per oral.

compared using a one or two-way ANOVA followed by a post-hoc Dunnett's test for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of PBA on the glycation of albumin. When the fluorescence intensity of the control samples was defined as 100%, the fluorescence intensities measured when treated with 0.156, 0.625, 2.5, 10 and 40 mM PBA were 92.6, 91.1, 86.6, 73.4 and 57.9%, respectively. The fluorescence intensities measured when treated with 0.156, 0.625, 2.5, 10 and 40 mM aminoguanidine, a known anti-glycation agent, were 85.9, 64.5, 46.0, 18.7 and 2.2%, respectively (Fig. 1).

Effect of PBA on the glycation of collagen. When the fluorescence intensity of the control samples was defined as 100%, the fluorescence intensities when treated with 0.156, 0.625, 2.5, 10 and 40 mM PBA were 88.0, 92.4, 91.2, 79.2 and 63.1%, respectively. The fluorescence intensities associated with aminoguanidines were 74.5, 71.5, 54.9, 21.7 and 3.9%, respectively (Fig. 2).

Effect of PBA on glycation in KK mice. The effect of oral administration of PBA on KK mice was monitored for

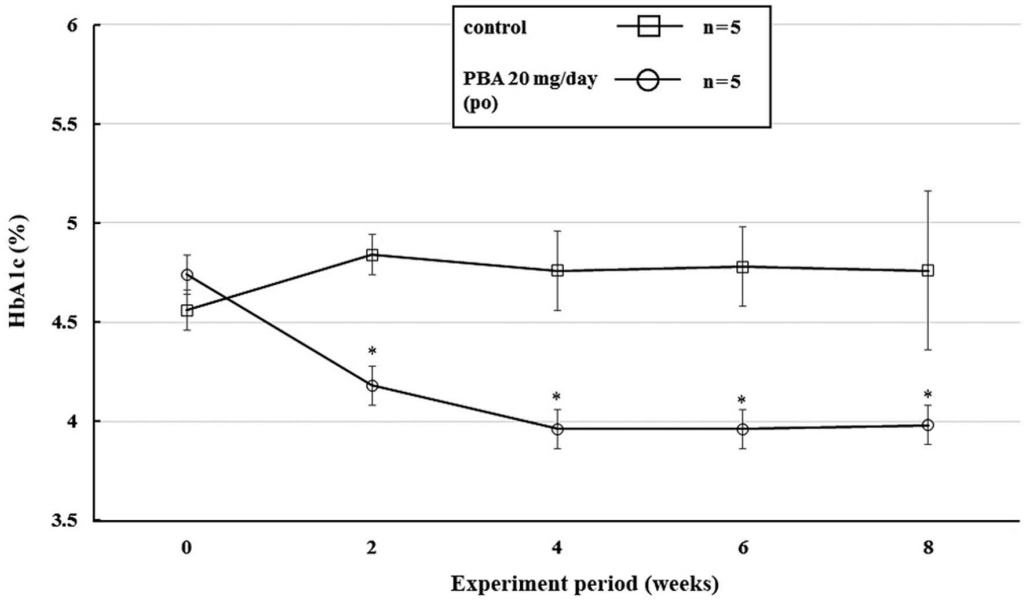

Figure 4. Inhibitory effect of PBA on the increase in blood HbA1c in KK mice. The vertical axis shows the blood HbA1c level as a percentage for each group and the horizontal axis shows the number of weeks of the study elapsed. Data are presented as the mean ± standard error of the mean, and were analyzed using a two-way ANOVA. n=5. *P<0.01 vs. Control. Control, H_2O; PBA, sodium 4-phenylbutyrate; po, per oral.

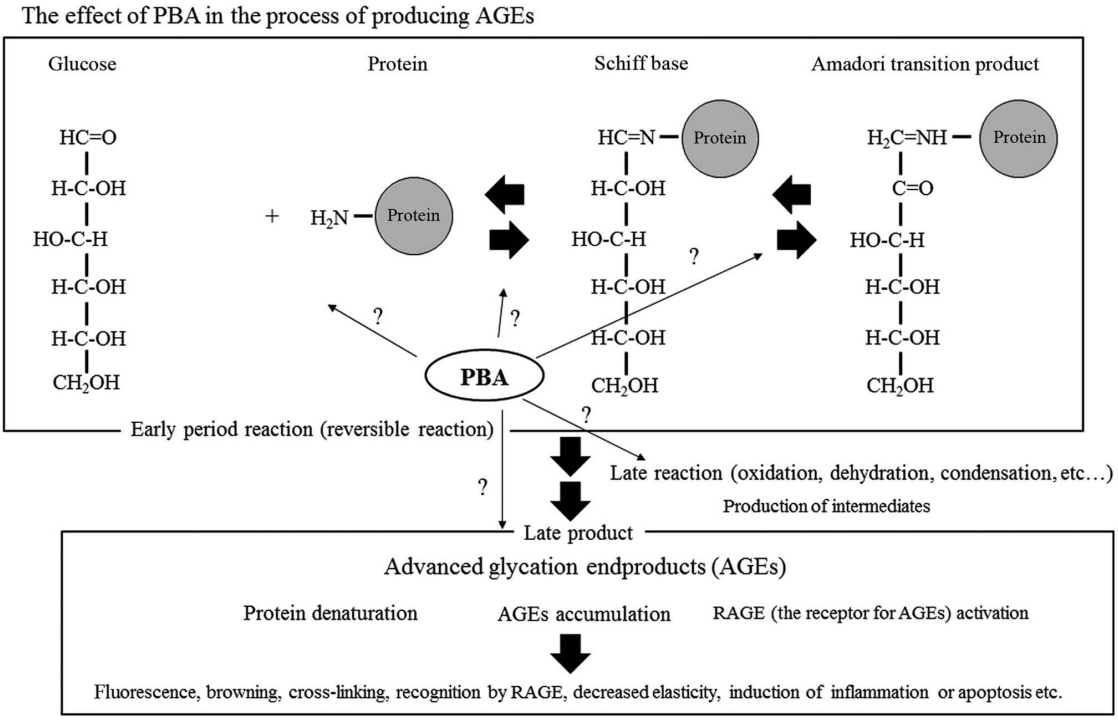

Figure 5. Effect of PBA on the production of AGEs. PBA, sodium 4-phenylbutyrate; AGE, advanced glycation end-product.

8 weeks. In the PBA-treated group, the development of glycosuria was delayed, and the weight gained as well as HbA1c levels were lower when compared with the control group. No glycosuric PBA-treated mice were identified after 1 week, whereas 2 mice in the control group were glycosuric after the same time period. At the end of the experiment, 2 glycosuric PBA-treated mice were identified, whereas all the mice in the control group were glycosuric (Fig. 3). The mean body mass increase in the PBA group was lower than that in the control group at every week of the study, and the difference in the mean body mass of the groups was ≤3.1 g during this period. The results of the two-way ANOVA were as follows: Treatment (PBA or control): $F(1, 4)=26.7$, $P=0.0066$; Time (weeks): $F(8, 32)=74.0$, $P<0.0001$; and Treatment x Time interaction: $F(8, 32)=4.6$, $P=0.0007$ (Fig. 3). In addition, the blood HbA1c levels were 3.96% after 4 weeks, and remained ~3.9% in the PBA-treated group until the end of the study. The results of the two-way ANOVA were as follows: Treatment (PBA or

control): F(1, 4)=18.1, P=0.0131; Time (weeks): F(4, 16)=1.1, P=0.3657; and Treatment x Time interaction: F(4, 16)=3.4, P=0.0329 (Fig. 4). There were no significant differences in food intake between the groups during the experiment (control, 5.3 g/day/mouse; PBA group: 5.4 g/day/mouse).

Discussion

In the present study, the effects of PBA on protein glycation were assessed. The effects of PBA on non-enzymatic glycation *in vitro* were first determined. The effect of PBA on the glycation of albumin *in vitro* was assessed as it is the principal serum protein, and its effect on collagen was assessed due to the possibility that it may be co-administered with cosmetics, supplements or pharmaceutical products that also have effects on collagen. Collagen is the primary structural protein in the extracellular matrix in various connective tissues. Therefore, the suppression of collagen glycation can be expected to be applied as a supplement or cosmetic with a beauty effect (23,24). Incubation of PBA in a solution of albumin in DPBS and α-glucose reduced the fluorescence intensity by up to 42.1% compared with the control, suggesting that PBA reduced the glycation of albumin. The effect of PBA on the glycation of collagen was then assessed using a commercially available kit, and found that there was a 36.9% reduction in collagen glycation, suggesting that PBA also reduced the glycation of collagen. Thus, through these experiments, it was confirmed that the addition of PBA reduced the glycation of albumin and collagen *in vitro*, and the saccharification of hemoglobin *in vivo*. The glycation reaction is complex, and the *in vitro* conditions (incubation in 0.2 M glucose at 60˚C for 40 h) were more extreme than those observed *in vivo*, in order to rapidly generate AGEs. The quantity of AGEs produced when the human serum albumin (HSA) (8 mg/ml) and glucose (0.2 M) are incubated at 60˚C for 40 h corresponds to ~60 days at 37˚C (23). However; there are limitations to this approach. The possible anti-glycation effects of PBA was evaluated using an established *in vitro* anti-glycation evaluation method. The levels of protein glycation should ideally be evaluated by measuring the residual unreacted amino/guanidino groups of lysine, arginine, and N-terminal amino acids, and thus, a modified approach will be used in subsequent experiments. Furthermore, the *in vivo* conditions are complicated by various other factors. Although comparison of *in vitro* and *in vivo* experiments are not easy, it is possible that PBA administration may inhibit the glycation of albumin and collagen *in vivo*. Previous reports have shown that PBA binds to albumin (25,26); therefore, it is hypothesized that the binding of PBA to albumin, the most abundantly present serum protein, may reduce glycation. The binding of PBA, and its inhibitory effect on the glycation of albumin and collagen in more detail will be assessed in future studies.

Having established the effects of PBA on protein glycation *in vitro*, KK mice, which develop diabetes, a disease that involves glycation (27), were administered PBA for 8 weeks. HbA1c levels were assessed as this is used as a key index of glucose control in diabetes (28). The results showed that there was a reduction in HbA1c levels in PBA-treated mice, suggesting that PBA may have an anti-saccharification effect as HbA1c is glycated hemoglobin. In the PBA-treated group, the development of glycosuria was delayed, and the weight gain and HbA1c levels were lower compared with the control group, but there was no significant difference in food intake between the groups during the experiment (~5 g/day/mouse). These results suggest that it is necessary to evaluate other markers, such as carboxymethyl lysine (CML), carboxymethyl arginine, pentosidine and pyraline. However, as HbA1c is a glycated stress marker, PBA administration is likely to reduce glycation *in vivo*.

In vivo glycation and the formation of AGEs can also be induced by several other carbonyl molecules; therefore, the levels of major protein glycosylation markers, such as CML, glucospine, pentosidine and glucoalbumin (a glycated protein) should be directly measured in future studies. The safety of PBA at the administered doses has been shown to be safe and is the established amount administered to humans in existing drug preparations (such as Buphenyl: 450-600 mg/kg daily, divided into 3-6 doses and orally administered with or immediately after meals or nutritional supplementation) (14,15,19-21) (and Buphenyl interview form). In addition, it is necessary to determine in detail at which stage of AGE production PBA exhibits its effects; for example, the effect of PBA on reversible reactions (such as Schiff base formation and Amadori transition formation) should be investigated. Furthermore, the effects of PBA on oxidation, dehydration, condensation as well as other aspects of the late reactions, such as oxidative stress, inflammatory reaction and protein denaturation, should be determined. Figure 5 shows the action of PBA in a simplified glycation reaction system (Fig. 5).

In vitro results in the present study confirmed that PBA exhibited an inhibitory effect on albumin and collagen glycation. Furthermore, it was shown that HbA1c levels were reduced by PBA when administered to KK mice. The present study is the first to show the effects of PBA on albumin and collagen glycation *in vitro*, as well as its *in vivo* effects on HbA1c levels, to the best of our knowledge. However, the reduction in weight gain *in vivo*, or the mechanism by which PBA affects HbA1c levels in the absence of an effect on blood glucose concentration cannot be explained, and thus requires further study. It is hypothesized that the glycation of albumin, collagen and other proteins also occurs in mice. A previous study showed that human serum albumin and PBA bind to each other, thus PBA may bind to albumin and inhibits its binding to glucose at an early stage in the process of glycation (25,26). As the process of saccharification *in vivo* is complex, it is first necessary to identify measurable AGEs and compare the levels of glycation of each in the control and PBA-administered mice. Additionally, the strength of the interaction between PBA and albumin will be assessed using surface plasmon resonance in future studies. However, it should be noted that the PBA-treated mice did not exhibit increased urinary albumin concentration levels compared with the control mice (data not shown).

In conclusion, PBA may limit the aging process and delay the development of lifestyle-related and other chronic diseases, such as diabetes, atherosclerosis, hyperlipidemia, cardiovascular diseases, cerebrovascular disorders, chronic renal failure and neurodegenerative diseases, which are characterized by the glycation of proteins. Reducing the prevalence of lifestyle-related diseases, which are increasing annually worldwide, may substantially reduce the economic burden on healthcare systems. Although it is necessary to elucidate the mechanism by which PBA reduces glycation in more detail, the method of

administration and the side-effects of PBA are well established, as it is a currently used therapeutic. Therefore, administering PBA clinically for alleviating aging and lifestyle related disorders may be an additional use in the relatively near future.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

KO and MN conceived the study and drafted the manuscript. KO acquired the data. KO and MN analyzed the data and revised the manuscript. Both authors read and approved the final manuscript.

Ethics approval and consent to participate

The animal protocol was approved by the Experimental Laboratory Animal Committee of Fukuoka University (approval no. 1909069).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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